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Influence of various forms of iron on growth of *Chlorella vulgaris* Beijer culture

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Abstract. A comparative study of the toxic effect of magnetic nanoparticles of iron oxide (MNPs) and ions of bivalent and trivalent iron in the composition of sulfates on the growth of an intensive culture of *Chlorella vulgaris* Beijer is provided. The high sensitivity of the introduced biotest is shown for the case of a standard toxicant (potassium dichromate). Using the probit-analysis, and semi-extincting specific growth rate of EC50 culture concentrations were established in four-day experiments, which amounted to 1.3 ± 0.2 mg/l for potassium dichromate, 0.8 ± 0.1 mg/l for ferrous iron, 2.0 ± 0.2 mg/l for trivalent l and for MNPs iron oxide – 13 ± 1 mg / l. The probit analysis features and the significantly lower semi-suppressing specific growth rate of the culture in experiments with MNPs compared with ionic iron suggests a possible manifestation of other forms of exposure that are not related to the mechanism of chemical intoxication.

1. Introduction

Magnetic nanoparticles (MNPs) of natural and anthropogenic origin become a subject of special interest in the recent years [1-2]. In part, this interest is related to the increasing number of their biomedical applications in very different specific areas from environmental control and regenerative medicine to drug delivery, hyperthermia and biosensing [3]. Extended discussion on possible biomedical applications was focused onto physical properties of MNPs but biocompatibility tests in combination with careful characterization of the water-based suspensions of MNPs were under focus to the less extent. The excess content of iron ions in both natural waters and domestic water supply systems is now becoming an increasingly common phenomenon that can have a negative impact on humans and on natural populations of aquatic organisms. The greatest danger is sewage and sludge from industries related to the processing of iron-containing products.

According to the hygienic standards maximum permissive dose (MPD) of chemicals in the water for drinking water or cultural and community water use applied for the water systems (introduced in 2003, Hygienic Standards 2.1.5.1315-03.154 [4]), 1 MPD of iron in any inorganic form is equal to 0.3 mg/l. At the same time, in a number of sources, including the standard for determining the degree of hazard of the materials NFPA-704 [5], there are significant differences in the toxicity of various iron compounds, as well as the greater toxicity of ferrous iron compared with trivalent. Nevertheless, the works devoted to a special study of this issue, as well as on the influence of other forms of iron (nanoparticles or nanorods) are practically absent in the literature.



This work is devoted to a comparative analysis of the toxic effect of iron oxide magnetic nanoparticles ($\gamma\text{-Fe}_2\text{O}_3$) and ions of bivalent or trivalent iron on the growth of microalgae *Chlorella vulgaris* Beijer culture.

2. Materials and methods

Iron oxide ($\gamma\text{-Fe}_2\text{O}_3$ maghemite) MNPs were prepared by the laser target evaporation technique (LTE). The formation of uniform spherical particles is performed in a circulating gas flow, which cools down the vapour and prevents particles from agglomerating [6].

Structure of LTE MNPs was studied by X-ray diffraction method (XRD) using DISCOVERD8 Bruker equipment and Cu-K α radiation with accurate fit for the fullprofile and the Miller indices identification. The average size of MNPs was estimated using the Scherrer formula [7]. Exact chemical composition of obtained MNPs was carefully determined by the Redox titration and the XRD lattice period analysis provided at a time.

The shape of the particles was evaluated and distribution of the particle size was calculated from the data of transmission electron microscopy (TEM) using analysis of the images of about 2000 nanoparticles. Electron microscopy studies performed using a JEOL JEM-2100 microscope (operating voltage was 200 kV). For the observations previously de-aggregated MNPs were spread onto Cu conducting grid.

Magnetic MNPs for biological applications can be used only in the form of water-based ferrofluids. Therefore corresponding suspension was prepared: air-dried MNPs were deaggregated by ultrasound treatment for 30 min using Cole-Parmer CPX-750 processor operated at 250 W with 5 mM sodium citrate water solution. The remained aggregates were eliminated by centrifuging. Magnetic measurements of the air-dry MNPs were carried out with SQUID-magnetometer. The object of the biological study was the algological pure culture of the microscopic green alga *Chlorella vulgaris* Beijer, in particular Strain IRK-A46 (isolated from the soils of Kazakhstan region. It was taken from the collection of algae cultures SIFIBR SB RAS, IRK-A (provided by doctor of biological sciences I.N. Egorova) [8].

Under laboratory conditions (intensive growing conditions), *Chlorella vulgaris* was cultivated in a thermoluminostat device with continuous illumination by fluorescent lamps (2500 Lux) and a constant temperature of 28 ° C in 100 ml flat-bottomed flasks using Prath artificial nutrient medium. The culture of algae from the mother suspension was sown onto fresh nutrient medium in concentration of about 0.7-0.9 million cells/ml and afterwards it grown for 7-10 days before reseedling.

Toxicological experiments were performed with: potassium bichromate ($\text{K}_2\text{Cr}_2\text{O}_7$), as a standard toxicant with a well-studied effect on green algae, in concentrations of 0.5; 1.0; 2.0 and 4.0 mg l [9]; ferrous ions from an aqueous solution of $\text{FeSO}_4 \times 7\text{H}_2\text{O}$, in concentrations of 0.015 to 1.5 mg/l, calculated for Fe^{2+} cations (that is, from 1/20 to 5 MPD for Fe^{2+} ions); ferric ions from an aqueous solution of $\text{Fe}_2(\text{SO}_4)_3 \times 7\text{H}_2\text{O}$, in concentrations from 0.03 to 6.0 mg/l, calculated for Fe^{3+} cations (that is, from 1/10 to 20 MPD for Fe^{3+}); suspensions of iron oxide nanoparticles ($\gamma\text{-Fe}_2\text{O}_3$) in concentrations from 0.9 to 30 mg/l (i.e., from 3 to 100 MPD by iron).

From the master batch solution of the toxicant, solutions of a given concentration were prepared by adding it to the culture flasks at the beginning of the toxicological experiment. The volume of liquid in the cultivation flasks was 100 ml. It was made up of the volume of the nutrient medium, the volume of the algae suspension from the mother culture in the exponential growth phase to an initial planting density of about 0.8 million cells/ml and the toxicant solution.

The main indicator for assessing the toxic effect of substances was the increase in the number of *Chlorella vulgaris* cells in the experiment compared with the control case. The density of the culture was estimated every 2 days throughout the entire time of the experiments (up to 10 days) by direct counting of cells in the Goryaev chamber with further conversion of the average counting results into culture density units (ppm/ml).

The second employed technique was the method of measuring the transmitted light absorption coefficient using laboratory photometer KFK-3. The measurement was made in relative absorption

units (relative units) at a wavelength of transmitted light $\lambda = 450$ nm (in the region of the blue absorption maximum for the sum of chlorophylls "a" and "b"). These measurements were performed in order to quickly estimate the concentration of algae in the sample, as between this indicator and the instrument readings for the intensity of absorption a strong positive correlation was found (correlation coefficient of 0.98). All experiments were performed according to the described method in 4-6 biological replicates, the results and graphs show the average values of the indicators. Calculations (finding the average, the average error and the student coefficient) were performed using standard statistical methods [10].

For an integrated assessment of the intensity of reproduction of algae, the specific growth rate (μ , 1/day) was calculated in accordance with equation:

$$\mu = \frac{\ln\left(\frac{C_t}{C_o}\right)}{t} \quad (1)$$

where C_t и C_o – cell concentration in the exponential (logarithmic) phase of culture growth at the beginning and end of the experiment, t is the time of the experiment (4 days).

For a comparative assessment of the sensitivity of algotest to the action of all toxicants, the probit-analysis method most effective was used [11-12]. In all experiments, the units were estimated by the degree of suppression of the specific growth rate of the culture (μ , 1/day, expressed in % of the value corresponding to control sample) 4 days after the start of the experiment, when the manifestation of the phase of primary disorders was usually the most pronounced [11]. For comparison of the degree of toxicity of substances, particular concentrations that suppressed the specific growth rate by 50% of the value in the control variant on the 4th day of the experiment (EC50) were found. For this purpose the unified graphical method of probit analysis was used.

Considering that the experiments were carried out under strictly controlled conditions according to the tested method, the experimental error usually did not exceed 15% of the average value.

3. Results and discussion

Let us compare the values of weighted average diameters of MNPs obtained by XRD technique and TEM: they were in a quite reasonable agreement both close to 15 nm (Figure 1). Chemical composition of MNPs was determined by Redox titration and the lattice period analysis provided by XRD. The lattice period ($a = 8.358 \pm 0.005$ Å) was somehow higher than the lattice period of $\gamma\text{-Fe}_2\text{O}_3$ but close to it. Magnetic measurements of thermomagnetic curves and hysteresis loops confirmed that MNPs state was close to a superparamagnetic (low coercivity of about 20 Oe and saturation magnetization of 35 emu/g at room temperature) with blocking temperature close to the room temperature.

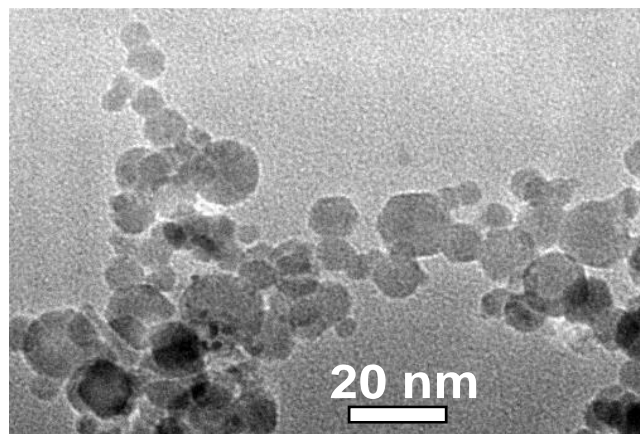


Figure 1. Magnetic nanoparticles obtained by laser target evaporation technique at the Institute of Electrophysics UD RAS (Ekaterinburg, Russia). Transmission electron microscopy.

In the present work we used Prath nutrition medium, as the necessary conditions for conducting toxicological experiments are relatively poor with respect to the elements mineral amount in nutrition medium. The culture of *Chlorella vulgaris* in intensive growing conditions appears to be for an approximately 7 days in an exponential growth phase, reaching following number of cells from 0.8 to 10.0-11.0 parts per million/ml (Figure 2).

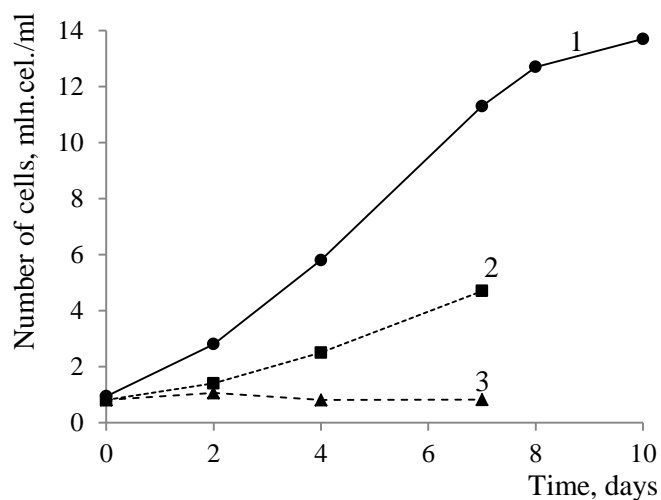


Figure 2. Growth dynamics of an intensive grows culture of *Chlorella vulgaris* Beijer on Prath's medium in the control (1) and action of potassium dichromate $K_2Cr_2O_7$ at a concentration of 1.0 mg/l (2) or 4 mg/l (3).

With further cultivation up to 10 days, the culture went into a phase of slow growth, which can probably be attributed to the exhaustion of nutrients and the toxic effect of the metabolic products of the cells themselves in a dense culture. In this regard, the duration of subsequent experiments did not exceed 7 days.

The most important indicator of the effectiveness of the applied biotest is its relatively high sensitivity to the action of toxicants, which is assessed in relation to any standard biotest. As such a standard, one can focus on a biotest with another green alga (*Scenedesmus quadricauda*) and potassium dichromate [9].

There was a significant lag in the growth of an intensive culture of chlorella compared with the control series in the conducted toxicological experiments. The lag parameters depended on the introduced concentration of potassium dichromate from 0.5 to 4 mg/l. On the second day of the experiment, the percentage reduction of 50-object. Thus, the ionic forms of iron showed almost the same and relatively low compared with potassium dichromate specificity of action on the growth of *Chlorella vulgaris* culture, since probit-analysis graphs are hollow with respect to the X axis and indicate a rather wide range of effective concentrations, although the toxicity of ferrous iron was about 2.5 times higher than the toxicity of trivalent iron. 60% was observed in the number of cells of the experimental sample compared with the number of cells in the control. It was observed in the range of concentrations of potassium dichromate of 1.0-2.0 mg/l. It is known that if the obtained concentration of $K_2Cr_2O_7$ for 48 hours of exposure causes a decrease in the number of algae by 50% (EC_{50} for 48 h), while it is in the range of 1.3-2.5 mg/l, then the algae culture is suitable for biotesting [9].

This confirms the suggestion that the intensive *Chlorella vulgaris* culture tested on potassium bichromate and the strain IRK-A46 has a sufficient sensitivity and can be used for the high quality biotest. The optimum duration of toxicological experiments should be four days when the cultures are in the active exponential growth phase, and the primary decompensation phase [11] is quite vividly shown to calculate the specific growth rate as the most integral indicator characterizing the growth of cultures in experimental and control cases.

With the use of a proven technique, the effect of Fe^{2+} and Fe^{3+} ions and MNPs of iron oxide on the growth of chlorella culture was studied. The development of a toxic effect for all forms of iron was

similar to what that one what was observed under the action of potassium bichromate: the lag of growth of algae in the experiment compared with the control was most pronounced on the 3rd or 4th day of the experiment. In addition, the degree of suppression increased with increasing concentration of toxicants.

Minimum lethal concentrations in weekly experiments were for Fe^{2+} ions as high as 1.5 mg/l (5 MPD), for Fe^{3+} - about 6 mg/l (20 MPD), and for MNPs of maghemite - more than 30 mg/l (100 MPD). It is important to mention that concentrations below 1 MPD for both types of iron ions had a significant stimulating effect on the growth of *Chlorella vulgaris* culture. We were not able to detect similar effect for MNPs.

In order to obtain more accurate comparative characteristics of the toxicity of the substances under consideration, we used the method of graphical determination of the so-called semi-suppressive specific growth rate (EC_{50}) of the culture in the experiments and compared obtained results with the results for the control culture on the 4th day of the experimental study.

Figure 3(a) shows that for potassium dichromate, the half-increasing growth concentration was $\text{EC}_{50} = 1.3 \pm 0.2$ mg/l. The obtained result confirms the above mentioned conclusion about the sufficient sensitivity of the introduced biotest based on an intensive culture of *Chlorella vulgaris* for the rapid assessment of the toxicity of water pollutants.

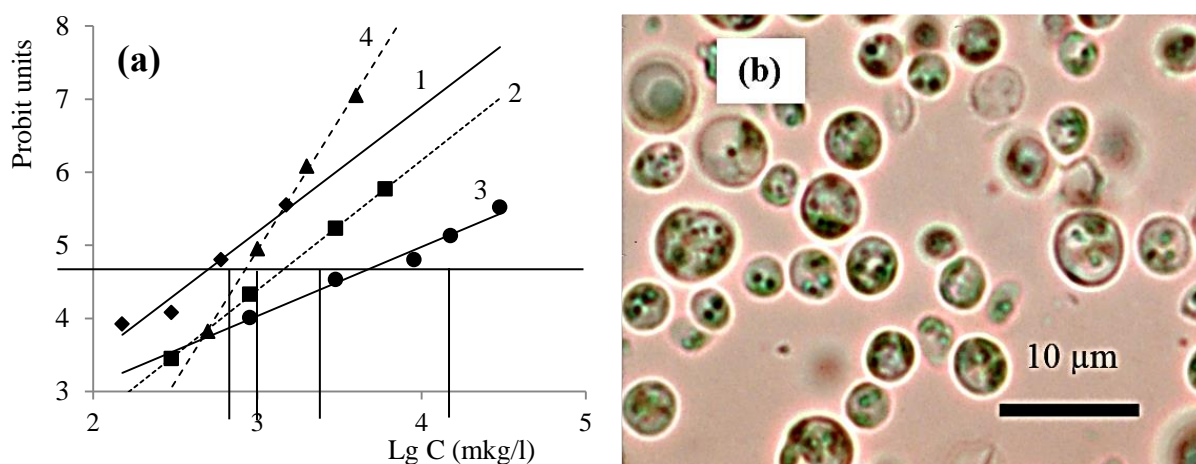


Figure 3. (a) Results of probit-analysis of the action of toxicants on the growth of *Chlorella vulgaris* Beijer culture (by suppressing specific growth rate on the 4th day of the experiment): Fe^{2+} (1); Fe^{3+} (2); Fe-nanoparticles (3); $\text{K}_2\text{Cr}_2\text{O}_7$ (4). (b) General view of *Chlorella vulgaris* cells with $\gamma\text{-Fe}_2\text{O}_3$ maghemite nanoparticles inside after 7 days of incubation (optical microscopy).

Fe^{2+} ions turned out to be significantly more toxic to green algae compared to Fe^{3+} ions. The semi-inhibiting concentrations increased as follows: for ferrous iron, $\text{EC}_{50} = 0.8 \pm 0.1$ mg/l (about 2.6 MPD), and for trivalent iron - $\text{EC}_{50} = 2.0 \pm 0.2$ mg/l (about 6.7 MPD). Potassium bichromate is a more specific toxicant for green algae, compared with both ionic forms of iron, since its range of action is narrower. The probit analysis chart is located more steeply to the concentration axis (axis X), which is typical for the action of toxicants that are highly specific. The effect of iron oxide $\gamma\text{-Fe}_2\text{O}_3$ maghemite MNPs was significantly different from the action of ionic forms of iron on the test culture. The semi-extinct concentration of MNPs, for the estimation of the parameters of the growths of *Chlorella vulgaris* cell culture was: $\text{EC}_{50} = 12.6 \pm 0.2$ mg/l (about 42 MPD).

The exceptionally shallow probit analysis graph and almost 6-10 times smaller compared with ionic forms of iron EC_{50} value in experiments with MNPs (Figure 3(a)) suggest the possible manifestation of other forms of exposure that are not associated with chemical intoxication. For

example, they could be such forms as mechanical disturbances in *Chlorella vulgaris* cells, connected with the penetration of nanoparticles inside the living cells and their agglomeration.

This assumption was confirmed by experimental micrographs, in which collapsed chromatophores and a large number of optically dense inclusions of a rounded shape were clearly visible. Possibly they were located inside the lysosomes with a high content of iron nanoparticles inside (Figure 3(b)). The understanding of possible damage contribution and mechanisms requires further careful research.

4. Conclusions

For the relatively poor in amount of elements Prath mineral nutrient medium, the intensively growing culture of *Chlorella vulgaris* used for the biotest can last for up to 7 days in the exponential phase, showing a relatively high specific growth rate ($\mu = 0.5 \pm 0.1$ 1/day).

Experiments on a standard toxicity-well-studied toxicant (potassium dichromate) showed a rather high sensitivity of the biotest proposed here for the intensive culture of *Chlorella vulgaris*. Detected in two-day experiments the semi-extinct concentration was about 1.0 - 2.0 mg/l, and the inhibitory specific growth rate in four-day experiments, for 50% decay was observed for the concentration $EC_{50} = 1.3 \pm 0.2$ mg/l.

A significantly (approximately 2.5 times) greater toxicity of ferrous iron ions was found in comparison with trivalent iron ions for intensive *Chlorella vulgaris* culture. The semi-inhibiting concentrations increased as follows: for ferrous iron ions, $EC_{50} = 0.8 \pm 0.1$ mg/l (about 2.6 MPD), and for trivalent iron ions - $EC_{50} = 2.0 \pm 0.2$ mg/l (about 6.7 MPD).

Both forms of iron ions (Fe^{2+} and Fe^{3+}) showed practically the same and relatively low compared with potassium bichromate specificity of action on the growth of *chlorella* culture, conclusion made on the basis of the probit-analysis plots for a rather wide range of concentrations.

The flat graph of probit analysis and almost 6-10 times smaller than the ionic forms of iron value of EC_{50} in the experiments with iron nanoparticles (12.6 ± 0.2 mg/l) suggests possible existence of the other forms of exposure that are not associated with chemical intoxication, for example, by mechanical disturbances of the *Chlorella vulgaris* cells as a result of MNPs penetration inside of them.

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